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# UTILITY PATENT APPLICATION TRANSMITTAL

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## APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

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### Modification of Plant Fibres

This invention relates to the modification of the morphology of plant fibre cells. The invention is exemplified by methods of using genetic constructs for the modification of, in particular, but not exclusively, *Eucalyptus* fibres, for example.

The primary product of the forestry industry is considered to be wood, although more fundamentally it could be defined as fibre. The industry supplies a wide range of feedstocks to the solid wood and pulp/paper industries who produce a multiplicity of products. The forester must therefore seek to cater for the competing needs of these industries, and even within the individual industries, there is a range of different requirements. For example, different paper grades require different qualities in the starting material.

Forestry-based operations depend upon a balance between the capability of the forester to supply the processor with fibre having specific properties, and the ability of the processor to modify his process and so accommodate the available feedstock. The design and operation of processing plants are influenced by the wood (fibre) properties of the feedstock.

Notwithstanding these specific demands, fibre uniformity and strength are common requirements for most industrial uses, and hence the fibre supplied by the forester must be capable of delivering these properties to the processor.

In pulp manufacture, for example, strength characteristics are determined in part by fibre length. Increased fibre length leads to the production of paper with increased strength. Bond strength is attributed to contact between the fibres and the adhesion capabilities of the surfaces, which are dependent upon fibre length, perimeter and coarseness. Also, during the manufacturing process, increased fibre length increases the strength of wet webs enabling easier handling (Seth, 1995).

However, long fibres are not desirable for all applications. In some cases, shorter fibres are preferable, such as in the production of smooth-surfaced papers.

Fibre properties differ between species, and consequently particular species have been limited historically to particular applications. Fibres from hardwood species are generally much shorter than those from softwoods. This results in the production of pulp and paper with desirable surface characteristics such as smoothness and brightness, but with low strength characteristics. In practice, where a single species providing fibre with an appropriate combination of characteristics has not been available, the mixing of long

and short fibres from different species is used. If a single source were available, possessing the desirable characteristics plus optimal fibre length, this would be of great benefit to the processor. Some common species and their fibre lengths are exemplified in Table 1 below.

Table 1

Fibre Lengths of Various Tree Species

Species	Fibre Length (mm)
Loblolly Pine	3.5 - 4.5
Western Hemlock, Western Spruce	2.5 - 4.2
Southern Hardwood	1.2 - 1.4
Northern Hardwood	1.0 - 1.2
<i>Eucalyptus</i>	0.8 - 1.0
White Oak	0.59
Sweetgum	0.48
Aspen	0.35

*Eucalyptus* trees represent the largest sources of fibres used globally in the paper industry (Bamber 1985; Ranatunga, 1964), and world-wide, there are an estimated ten to fifteen million hectares of land planted with *Eucalyptus* (Verhaegen and Plomion 1996). The major advantage of Eucalypts is their very high growth rates and ability to grow in a wide range of conditions, both tropical and temperate.

However, *Eucalyptus* fibres are significantly shorter than those from other, once more popular, sources of fibre such as pine. Thus papers that are made from *Eucalyptus* pulp are often weak and usually require reinforcement with longer fibres from other sources increasing the production costs. If trees could be produced with longer fibres, this would be a considerable advantage to the paper industry, increasing the quality of the raw materials for pulp and paper synthesis.

Through tree breeding it is possible to achieve some modification of fibre characteristics. For example, interspecific triploid hybrids of poplar have been developed which have longer fibres than the parental species.

Genetic variation in fibre properties is also evident within species. Fibre characteristics are controlled by a complex set of genetic factors and are not easily amenable to classical breeding methods. Therefore, existing genetic variation has not been exploited significantly in tree breeding programmes. Whilst knowledge is now being accumulated on the heritability of wood properties, previously these were not often considered as important as growth characteristics and were sometimes sacrificed in pursuit of the latter. In some instances, growth rate is negatively correlated with fibre characteristics, though this does not always hold true (e.g. in Eucalypts), and breeding efforts are now being made to capture the benefits of both.

In many cases fibre properties are sufficient for the end product, and improvement is considered unnecessary. For example, increasing fibre length beyond 2mm causes little increase in tear strength or tensile strength, and many softwood fibres are commonly around 3mm long, i.e. greater than the minimum for desired strength. However, fibres in juvenile wood tend to be shorter and there is an increased usage of juvenile material through a reduction in rotation times. Hence, there is scope for improvement even in those species which commonly yield long fibres.

From the perspective of the pulp and paper industry, fibres are specific types of plant cell walls that have been subjected to a range of treatments to remove all contents and most non-cellulosic wall components (Stewart *et al*, 1994). In woody plants the fibres are made up of dead cell wall material. In order to produce longer fibres it is necessary to have longer living cells during growth, before fibre formation.

The cell wall can be envisaged as a complex network of cellulose microfibrils linked together by noncovalent interactions with matrix polymers (Carpita and Gibeaut, 1993). The microfibrils are coated by a mixture of hemicelluloses which form extensive hydrogen-bonded interactions with the surface of the microfibrils. Coextensive with this is another

network formed from various pectins which are held together largely by ionic linkages (McQueen-Mason, 1995).

To allow cells to grow and enlarge the wall components must loosen to enable slippage of the polysaccharides and proteins within the matrix (Cosgrove, 1993). Extension of the cell is then driven by the internal turgour pressure of the cell, which is considerable. The degree of extension during cell growth is controlled by the mechanical properties of the cell wall, which result from their composition and from the orientation of wall fibrils and structural polymers.

The control of cell wall extension is closely regulated by the plant to facilitate growth control and morphogenesis. The ultimate agents of control are enzymes located in the wall itself. If plants express cell wall "loosening" enzymes in their walls, then it seems likely that these enzymes can regulate cell growth. Altered levels of expression can thereby cause increased or reduced cell growth and fibre length. Changes in cell wall texture may also be produced.

One class of cell wall proteins are the Expansins. Expansins induce the extension of plant walls, and at present are the only proteins reported with demonstrated wall-loosening activity. Expansins were first isolated from cucumber hypocotyl cell walls by McQueen-Mason *et al* (1992) and characterised by their ability to catalyse wall loosening in an *in vitro* rheological assay.

The mode of action of expansins is believed to be by weakening the noncovalent bonding between the cellulose and hemi-cellulose, with the result that the polymers slide relative to one another in the cell wall (Cosgrove 1996). The precise biochemical action of expansins is unclear, although it is known that their effects are not due to exoglycanase or xylogucan endotransglycosylase activity (McQueen-Mason *et al*, 1992, McQueen-Mason & Cosgrove, 1993). Expansins appear to disrupt hydrogen bonding between cellulose microfibrils and hemicelluloses. The process enables wall loosening without any degradation of the polymers or an overall weakening of wall structure during expansion. Consistent with this mechanism, expansins have been shown to weaken cellulosic paper, which derives its mechanical strength from hydrogen bonding between cellulose fibres (McQueen-Mason and Cosgrove, 1994).

Expansins are able to restore the ability of isolated cell walls to extend in a pH dependent manner (McQueen-Mason and Cosgrove, 1995) and may be responsible for the phenomenon of "acid growth" in plants (Shcherban *et al*, 1995). Expansin proteins have been characterised in cucumber hypocotyls (McQueen-Mason *et al*, 1992), oat coleoptiles (Li *et al*, 1993), expanding tomato leaves (Keller and Cosgrove, 1995) and rice internodes (Cho and Kende, 1997).

Expansin cDNAs have been isolated and characterised from a number of plants and it is now evident that expansins exist as a multi-gene family showing a high level of conservation between species. cDNAs with high degrees of homology have been identified from collections of anonymous Expression Sequence Tag (EST) cDNAs from *Arabidopsis* and rice. These EST cDNAs exhibit a high degree of homology at the level of protein sequence (60-87%) indicating that expansin structure is highly conserved (Shcherban *et al* 1995). Expansins show no sequence similarity to other known enzymes, although they do have sequence similarities to some pollen allergens (Shcherban *et al*, 1995). Recently Cosgrove *et al* (1997) have shown that pollen allergens from maize also possess considerable expansin activity.

If plants can be modified to over-express expansins in their walls, then it would be expected that these plants will exhibit a marked increase in cell extension or growth. Conversely, a reduction in the expression of expansins should lead to a reduction in cell growth. It is therefore surprising that constitutive expression of expansin in eucalypts results in a reduction in height and internode length.

One approach to modifying the expression of expansins is via the introduction of recombinant DNA sequences into the plant genome. Several methods can be used to introduce

foreign DNA into plant cells (see review by Weising *et al*, 1988; Miki and Iyer, 1990 and Walden 1994). *Agrobacterium tumefaciens*-mediated gene transfer is probably the most widely used and versatile of these methods (Walden, 1994).

Genetic modification experiments directed towards changing the wood and paper quality of trees has been investigated by other workers, particularly focusing on the lignin pathway in cells and lignin content in the final paper product (Hawkins and Boudet, 1994; Grima-Pettenati, *et al*, 1993; Poeydomenge *et al*, 1993; Boudet *et al*, 1995 and Hibino *et al*, 1994). The aim of the present invention differs in that it seeks to provide a means of controlling fibre growth and cell wall morphology.

An object of the present invention is to provide a method whereby trees can be modified to produce fibres of a desired length for specific applications. This will enable the forester to control the quality of his product. In addition it will enable the forester to produce a wide range of fibre types from a single or small number of species which can be selected as being ideally suited for cultivation in that particular site. This will result in both the economy of employing a single uniform silvicultural regime, and the flexibility of producing which ever type of fibre is required at a particular time.

The invention also provides a means of producing fibre of specific type from trees at particular periods in their growth cycle. For example, the production of long fibres from juvenile trees can be achieved, thereby accelerating the time to harvest of the crop.

This is achieved by firstly isolating and characterising expansin gene sequences from heterologous and homologous species and then reintroducing these genes into trees so as to alter expansin levels in the transgenic trees using the well known over-expression, co-suppression (described by DNAP in their European Patents Nos. 0465572 and 0647715) and anti-sense knockout strategies. This will lead to the cultivation of trees more suitable for paper production.

The present invention provides a nucleic acid coding sequence encoding a gene capable of modifying the extension of fibre cell walls, the nucleic acid coding sequence being one or more of SEQ.ID. Nos. 1-6 hereof.

The present invention also provides a method of transforming trees to modify the fibre characteristics in trees, the method comprising stably incorporating into the plant genome a chimaeric gene comprising a promoter and a nucleic acid coding sequence encoding a gene capable of modifying the extension of fibre cell walls, and regenerating a plant having an altered genome.

The present invention also provides trees having therein a chimaeric gene comprising a promoter and a nucleic acid coding sequence capable of modifying the extension of fibre cell walls.

The present invention also provides a chimaeric gene capable of modifying the extension of cell walls, said chimaeric gene comprising a promoter and a nucleic acid coding sequence encoding a gene capable of modifying the extension of fibre cell walls, said nucleic acid coding sequence being one or more of SEQ. ID. Nos. 1-6 or the cucumber Ex 29 coding sequence, or a sequence which has sufficient homology to hybridise to any one of SEQ. ID. Nos. 1-6 or cucumber Ex 29 under medium stringency conditions.

Preferably the chimaeric gene further comprises a terminator.

Constructs having the DNA structural features described above and trees incorporating such constructs and/or chimaeric genes according to the invention are also aspects of the invention.

Plant cells containing chimaeric genes comprising a nucleic acid coding sequence capable of modifying the extension of fibre cell walls are also an aspect of this invention, as is the seed of the transformed plant containing chimaeric genes according to the invention.

The chimaeric gene may comprise the nucleic acid coding sequence as it exists in the genome, complete with endogenous promoter, terminator, introns and other regulatory sequences, or the nucleic acid coding sequence, with or without introns, may be combined with a heterologous promoter, terminator and/or other regulatory sequences.

The promoter may be a constitutive promoter, such as the cauliflower mosaic virus 35S promoter (CaMV35S), the cauliflower mosaic virus 19S promoter (CaMV19S) or the nopaline synthase promoter, a tissue specific promoter, such as the *rolC*, patatin or *petE* promoters, or an inducible promoter, such as *AlcR/AlcS*. Other suitable promoters will be known to those skilled in the art.

The nucleic acid sequence, or parts thereof, may be arranged in the normal reading frame direction, i.e. sense, or in the reverse reading frame direction, i.e. antisense. Up or down regulation of the activity of the expansin protein or gene encoding therefor using sense, antisense or co-suppression technology may be used to achieve alteration in the length of fibre cell walls.

Preferably the nucleic acid sequence encodes one or more of the class of proteins known as expansins. More preferably the nucleic acid sequence is derived from *Eucalyptus* or cucumber.

The nucleic acid sequence may advantageously be one or more of SEQ. ID. Nos. 1-6 hereof. Alternatively, the nucleic acid sequence may be the cucumber expansin sequence cucumber Ex29 (GenBank Accession No. U30382 - known as Cs-EXP1). The sequence is also described in Shcherabon *et al* (1995).

Alternatively, the nucleic acid sequence may be a sequence which has sufficient homology to hybridise to any one of SEQ. ID. Nos. 1-6 or cucumber Ex29 under medium stringency conditions (washing at 2x SSC at 65°C).

Preferably the nucleic acid sequence is an mRNA or cDNA sequence, although it may be genomic DNA.

Trees which may suitably be transformed using the inventive method include Eucalypts, Aspen, pine, larch.

The nucleic acid sequence may be introduced by any of the known genetic transformation techniques such as *Agrobacterium tumefaciens* mediated transformation, *Agrobacterium rhizogenes* mediated transformation, biolistics, electroporation, chemical poration, microinjection or silicon-fibre transformation, for example.

In order that the invention may be easily understood and readily carried into effect, reference will now be made, by way of example, to the following Figures, in which:-

Figure 1a is a diagrammatic representation of the coding sequence for cucumber Ex29 cloned between the cauliflower

mosaic virus 35S promoter and nos terminator in the vector pDE326;

Figure 1b is a diagrammatic representation showing the insert from Figure 1a between the EcoR I and Hind III restriction sites introduced into a modified Ti plasmid pDE 1001 to produce pDE/EXP29, and

Figure 1c is a diagrammatic representation showing the insert from Figure 1a between the EcoR I and Hind III restriction sites introduced into a modified Ti plasmid p35GUSINT to produce pATC/EXP29; and

Figure 2 is a diagrammatic representation showing an insert containing SEQ. ID. No. 1 between the EcoR I and Hind III restriction sites introduced into a modified Ti plasmid p35GUSINT to produce pATC/SEQ. ID. No.1.

#### EXAMPLE 1

##### Isolation of novel expansin sequences from *E. grandis* stem tissue

RNA extraction from cucumber hypocotyls. Seeds of cucumber (*Cucumis sativus* L., cv Burpee pickler, from A.W. Burpee, Westminster, Penn, USA) were sown on water-soaked capillary matting (Fordingbridge Growers Supplies, Arundel, W. Sussex, UK) in plastic trays (35cm x 25cm x 6cm) and germinated in the dark at 27°C. After 4 days the etiolated seedlings were harvested under green light by excising the upper 20mm of the

hypocotyl into liquid nitrogen and grinding to a fine powder in a pestle and mortar that had previously been chilled at -80°C. Total RNA was extracted in a hot phenol/lithium chloride buffer according to the procedure of Verwoerd *et al* (1989).

RNA extraction from *Eucalyptus grandis*. *E.grandis* seeds were sown on trays (35cm x 25cm x 6cm) of Levington's F2 compost (Levington Horticulture Ltd., Ipswich, Suffolk, UK) and germinated in a greenhouse (18-24°C, at a light intensity of approximately 10,000 lux, and 16 hours of daylight). After 8 weeks the seedlings were transferred to individual pots, and then repotted as necessary (approximately every 6-7 weeks). Growing stem tissue was harvested from the last 40-50mm of branch tips into liquid nitrogen. Immature leaves, usually the youngest two from growing branch tips, were also harvested directly into liquid nitrogen; roots were washed in several bowls of tap water, rinsed with distilled water and then growing tips were excised into liquid nitrogen. RNA was extracted as described by Pawlowski *et al* (1994) using a protocol especially modified for the extraction of RNA from plants containing high levels of phenolic compounds.

Poly(A<sup>+</sup>) mRNA isolation from total RNA extracted from *E.grandis* stem tissue. Poly(A<sup>+</sup>) mRNA was isolated from total RNA using either push (Stratagene, Cambridge, UK) or spin

oligo(dt) columns (Clontech Laboratories, Inc. CA., USA) and following the supplier's instructions and recommendations.

RT-PCR and Sequencing. The nucleic acid sequence of expansins show a considerable extent of divergence. However two regions with a reasonable degree of consensus were identified and used to synthesise two oligonucleotide primers of low complexity (see Table 2).

Total RNA was extracted from young stem tissue and Poly(A<sup>+</sup>) mRNA isolated using oligo(dt) columns as described above. 1 $\mu$ g of mRNA was used in a PCR experiment (50°C annealing temperature, 30 cycles, hot start) with the two expansin consensus primers and Taq DNA polymerase (Promega UK Ltd.).

Table 2

Sequence of Consensus Expansin Primers

Sequence (5' -3')

P.1 (SEQ. ID. No. 7) ATGGIGGIGCNTGYGGNTA

P.2 (SEQ. ID. No. 8) TGCCARTTYTGNCCCCARTT

**Key: Y=C or T, N=A or G or C or T, R=A or G, I=Inosine**

cDNA Library Construction. For first strand cDNA synthesis 1 $\mu$ g of mRNA was used in a reaction with 0.15 $\mu$ g OG1 oligo dt primers and AMV Reverse Transcriptase (9 units/ $\mu$ l, Promega UK Ltd., Southampton, UK).

The library was constructed in the Lambda ZAP II vector (Stratagene, Cambridge, UK), following the supplier's instructions.

Using the methods described, transformed clones were isolated by blue-white colony selection on agar plates following the methods described by the supplier (R&D Systems). Twenty white ("positive") colonies were selected and sequenced. Of these, six were identified as containing sequences that had similarities with other known expansin sequences using a basic BLAST search provided by NCBI. The putative transcripts were all around 450 bps in size (determined by PCR and gel electrophoresis). PCR products were sequenced using a forward primer and the sequences identified as SEQ.ID. Nos. 1-6 were obtained.

#### EXAMPLE 2

##### Northern Analysis

Total RNA was isolated from the stem, leaves and roots of *E. grandis* as described above. 6 $\mu$ g of RNA in 20 $\mu$ l DEPC H<sub>2</sub>O was denatured in a equal volume of denaturing solution (50% formamide, 2x TBE) and run on a standard 1.5% agarose gel at 75 volts for 200 min. RNA from the gel was transferred onto "Zeta-Probe" GT Genomic Tested Blotting Membranes (Biorad Laboratories, California, USA) by capillary transfer. Partial *E.grandis* expansin sequences generated by RT-PCR from stem

mRNA (as described above) were used for  $^{32}\text{P}$ -random prime labelling and hybridised to the transferred RNA following the membrane supplier's recommended methods (Biorad Laboratories).

### Example 3

#### Preparation of Exp29 transformation vector.

RNA extraction from cucumber hypocotyls. Seeds of cucumber (*Cucumis sativus* L., cv Burpee pickler, from A.W. Burpee, Westminster, Penn, USA) were sown on water-soaked capillary matting (Fordingbridge Growers Supplies, Arundel, W. Sussex, UK) in plastic trays (35cm x 25cm x 6cm) and germinated in the dark at 27°C. After 4 days the etiolated seedlings were harvested under green light by excising the upper 20mm of the hypocotyl into liquid nitrogen and grinding to a fine powder in a pestle and mortar that had previously been chilled at -80°C. Total RNA was extracted in a hot phenol/lithium chloride buffer according to the procedure of Verwoerd *et al* (1989).

Vector construction. The coding sequence for cucumber Ex29 (GenBank Accession No. U30382; known as Cs-EXP1, and Shcherban *et al* 1995) was generated by RT-PCR and cloned between the Cauliflower Mosaic Virus 35S promoter and nos terminator (see Figure 1a) into pDE326, a vector kindly donated by Dr. Jürgen Denecke of York University. After insertion of the Ex29 expansin sequence the inserts were

sequenced to check for correct in frame insertion by sequencing using a primer located within the 35S promoter region.

Inserts containing the 35S promoter, Ex29 sequence and nos terminator were cut between the EcoRI and HindIII restriction sites and inserted into modified Ti plasmids to produce transformation constructs. Two modified Ti plasmids were used: pDE1001 (Denecke et al, 1992 or Shcherban et al 1995) provided by Dr. Jürgen Denecke and p35GUSINT (Vancanneyt et al, 1990). The plasmids produced containing the insert were referred to as pDE/EXP29 (pDE1001 + Ex29) (see Figure 1b) and pATC/EXP29 (p35GUSINT + Ex29) (See Figure 1c), acknowledging the source of the plasmids.

Plasmids were transferred into *E.coli* by standard procedures; *E.coli* strains were grown on LB plates (incubated at 37°C and stored at 4°C) or in LB medium with the appropriate antibiotic for positive selection.

The constructs were introduced into *Agrobacterium* via direct DNA transformation or by tri-parental mating using the *E.coli* mobilisation function strain HB101 (pRK2013) (Figurski and Helinski 1979).

Two strains of *Agrobacterium tumefaciens* were used. A C58 strain (C58C1(pGV2260) Deblaere, R. et al 1985) kindly donated by Dr. Jürgen Denecke, and EHA105 (Hood et al 1993). *Agrobacterium* were grown on LB plates (incubated at 27°C and

stored at 4°C) or in LB medium with the appropriate antibiotic for positive selection.

*Agrobacterium tumefaciens* EHA105 pATC/EXP29 was deposited by Advanced Technologies (Cambridge) Limited of 210 Cambridge Science Park, Cambridge CB4 4WA, under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the purposes of Patent Procedure at the National Collection of Industrial and Marine Bacteria (NCIMB), 23 St. Machar Street, Aberdeen, Scotland on 25 August 1998 under Accession No. NCIMB 40968. The micro-organism is *Agrobacterium tumefaciens* : strain EHB105, containing pATC/EXP29. The cDNA for cucumber EX29 was inserted into disabled/disarmed pBIN19 (Bevan, 1984) with the 35S cauliflower mosaic virus promoter and nos terminator. The plasmid was then transferred into the *Agrobacterium* strain EHA105. The construct is useful for altering the extension of fibre cell walls.

#### EXAMPLE 4

##### Plant Transformation

Young leaves were dissected under sterile conditions, from approximately 4 week old *E.grandis* cultures grown in Magenta boxes (7cm x 7cm x 13cm) on LS media at 25°C, in a growth room in our tissue culture laboratory and used for *Agrobacterium*-mediated infection (Horsch, Fry, Hoffman,

Eichholtz, Rogers, and Fraley 1985). Inoculated tissue was left to co-cultivate for 4d on LS media (plus 20g/l glucose, 0.7% agarose, 0.01mM Zeatin a 1 $\mu$ M NAA) in diffuse light in a growth room, conditions as before. Transformants were selected on 50mg/l kanamycin and 250mg/l claforan.

Two constructs for plant transformation were prepared and introduced into two strains of *Agrobacterium*, C58 and EHA105 to produce C58 containing pDE + Ex29, C58 containing pATC + Ex29 and EHA105 containing pATC + Ex29. Each construct-containing strain was used to inoculate 400 leaves dissected from *E.grandis* tissue (on two separate occasions, each time inoculating 200 leaves).

The transformation experiments were repeated with a further 240 leaves, inoculated with EHA105 containing pATC + Ex29 to increase the amount of possible transformants obtainable.

From the original batch of inoculated tissue with EHA105, 25 plants were grown in the greenhouse and the properties of the shoots determined.

The introduction of the expansin coding sequence attached to the 35SCAmV promoter seems to have caused a reduction in the overall height of the plants from a mean control value of 603mm in the control plants to 546mm in the transformed plants. Of the survivors of the 25 plants, 4 control and 13 transgenic plants were included in this analysis. This

reduction in height is associated with a change in internode length as analysed in the table below. A Chi square analysis of the data in Table 3 indicates that the two populations of plants are significantly different at a value of  $P<0.01$ .

TABLE 3

Class of Internode Length (mm)	Number of Internodes in class		% of internodes in class	
	Control	Expansin	Control	Expansin
10	1	7	3	6
20	10	28	28	24
30	9	28	28	24
40	4	21	11	18
50	1	9	3	8
60	6	12	17	10
70	2	7	6	6
80	3	4	8	3
>80	01			

From the data it is clear that a modification in the level of expansin activity in the tree can be used to produce a required effect. In order to increase the growth it may be necessary to use down regulation technology, e.g. expression of the reverse or complementary strand of the expansin sequence, or a partial sense expansin sequence, in order to increase the fibre length.

EXAMPLE 5

Sequences SEQ. ID. Nos. 1-6 were each introduced into pATC in both orientations, i.e. antisense and sense orientation, and were used to transform Eucalypts and tobacco using the same methodology as described in Examples 3 and 4. Figure 2 shows the plasmid pATC/SEQ. ID. No. 1 in sense orientation, as a representative of the plasmids used in the transformation. Any suitable transformation vector can be used.

It was found that the introduction of the novel expansin sequences produced transformed plants different from the control plants.

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**BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE**

Advanced Technologies (Cambridge) Ltd  
Unit 210  
Cambridge Science Park  
Cambridge  
CB4 4WA

## INTERNATIONAL FORM

**RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT  
issued pursuant to Rule 7.1 by the  
INTERNATIONAL DEPOSITORY AUTHORITY  
identified at the bottom of this page**

I IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR.	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY:
<i>Agrobacterium tumefaciens</i> (EHA 105 pATC/EXP29)	NCIMB 40968
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by.	
<input type="checkbox"/>	a scientific description
<input checked="" type="checkbox"/>	a proposed taxonomic designation
(Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depository Authority accepts the microorganism identified under I above, which was received by it on 25 August 1998 (date of the original deposit) <sup>1</sup>	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depository Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: NCIMB Ltd.,  Address: 23 St Machar Drive, Aberdeen, AB24 3RY, Scotland.	Signature(s) of person(s) having the power to represent the International Depository Authority or of authorised official(s):   Date: 28 August 1998

Where Rule 6/4(d) applies, such date is the date on which the status of International Depositary Authority was acquired

**Form BP/4 (sole page)**

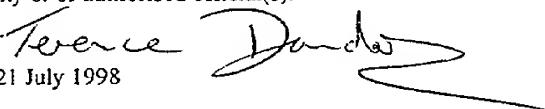
**BUDAPEST TREATY ON THE INTERNATIONAL  
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS  
FOR THE PURPOSES OF PATENT PROCEDURE**

Advanced Technologies (Cambridge) Ltd Unit 210 Cambridge Science Park Cambridge CB4 4WA	<b>INTERNATIONAL FORM</b>  <b>VIABILITY STATEMENT</b> issued pursuant to Rule 10.2 by the <b>INTERNATIONAL DEPOSITORY AUTHORITY</b> identified on the following page
---	---

**NAME AND ADDRESS OF THE PARTY  
TO WHOM THE VIABILITY STATEMENT  
IS ISSUED**

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name AS ABOVE Address:	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: NCIMB 40968
	Date of the deposit or of the transfer <sup>1</sup> . 25 August 1998
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on    26 August 1998    2. On that date, the said microorganism was:	
3 <input checked="" type="checkbox"/> viable 3 <input type="checkbox"/> no longer viable	

- 1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
- 2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.
- 3 Mark with a cross the applicable box.

IV CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED <sup>4</sup>	
V. INTERNATIONAL DEPOSITORY AUTHORITY	
Name: NCIMB Ltd , Address. 23 St Machar Drive, Aberdeen, A24 3RY, Scotland.	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):  Date: 21 July 1998 

4 Fill in if the information has been requested and if the results of the test were negative.

CLAIMS

1. A method of transforming trees to modify the fibre characteristics in trees, the method comprising stably incorporating into the plant genome a chimaeric gene comprising a promoter and a nucleic acid coding sequence encoding a gene capable of modifying the extension of fibre cell walls, and regenerating a plant having an altered genome.
2. A method according to Claim 1, wherein said nucleic acid sequence encodes one or more of the class of proteins known as expansins.
3. A method according to Claim 1 or 2, wherein said nucleic acid sequence is derived from *Eucalyptus* or cucumber.
4. A method according to Claim 3, wherein said nucleic acid sequence is one or more of SEQ. ID. Nos. 1-6 hereof or the cucumber expansin sequence known herein as cucumber Ex29 (GenBank Accession No. U30382), or a sequence which has sufficient homology to hybridise to any one of SEQ. ID. Nos. 1-6 or cucumber Ex29 under medium stringency conditions (washing at 2x SSC at 65°C).
5. A method according to any one of the preceding claims, wherein said nucleic acid sequence is an mRNA, a cDNA sequence or a genomic DNA.

6. A method according to any one of Claims 1-5, wherein said chimaeric gene is in accordance with any one of Claims 8-13.
7. A nucleic acid coding sequence encoding a gene capable of modifying the extension of fibre cell walls, the nucleic acid coding sequence being one or more of SEQ. ID. Nos. 1-6 hereof, or a sequence which has sufficient homology to hybridise to any one of SEQ. ID. Nos. 1-6 under medium stringency conditions.
8. A chimaeric gene comprising a promoter and a nucleic acid sequence encoding a gene capable of modifying the extension of fibre cell walls, said nucleic acid sequence being one or more of SEQ. ID. Nos. 1-6 hereof, or a sequence which has sufficient homology to hybridise thereto under medium stringency conditions.
9. A chimaeric gene according to Claim 6 or 8, wherein said chimaeric gene further comprises a terminator.
10. A chimaeric gene according to Claim 6, 8 or 9, wherein said chimaeric gene comprises said nucleic acid coding sequence as said nucleic acid sequence exists in nature, complete with endogenous promoter, terminator, introns and other regulatory sequences.
11. A chimaeric gene according to Claim 6, 8 or 9, wherein said chimaeric gene comprises said nucleic acid coding sequence, with or without introns, combined with a

heterologous promoter, terminator and/or other regulatory sequences.

12. A chimaeric gene according to any one of Claims 6 or 8-11, wherein said promoter is one of said group consisting of the cauliflower mosaic virus 35S promoter (CaMV35S), the cauliflower mosaic virus 19S promoter (CaMV19S), the nopaline synthase promoter, the *rolC*, patatin or *petE* promoters, or the AlcR/AlcS promoter.
13. A chimaeric gene according to any one of Claims 6 or 8-12, wherein said nucleic acid sequence, or parts thereof, is arranged in the normal reading frame direction or in the reverse reading frame direction.
14. A tree comprising a chimaeric gene according to any one of Claims 8-13.
15. A plant cell comprising a chimaeric gene according to any one of Claims 8-13.
16. A tree transformed according to the method of any one of Claims 1-7.
17. A tree according to Claim 16, said tree being a eucalypt, aspen, pine or larch.
18. A seed of a tree transformed according to the method of any one of Claims 1-7.

**ABSTRACT****Modification of Plant Fibres**

The invention relates to the isolation and characterisation of novel expansin gene sequences from heterologous and homologous tree species and re-introducing such novel genes into trees so as to alter expansin levels. Six novel genes have been identified. *Eucalyptus* has also been transformed using the cucumber EX29 sequence (GenBank, Accession No. U30382 - known as Cs-EXP1). A change in the plant height and internode length was observed compared with control plants.

SEQUENCE LISTING(1) **GENERAL INFORMATION**(i) **APPLICANTS:**

(A) NAME: Advanced Technologies (Cambridge) Limited  
 (B) STREET: Globe House, 1 Water Street  
 (C) CITY: London  
 (E) COUNTRY: England  
 (F) POSTAL CODE: WC2R 3LA

(ii) **TITLE OF INVENTION:**

Modification of Plant Fibres

(iii) **NUMBER OF SEQUENCES:**

8

(iv) **CORRESPONDENCE ADDRESS:**

(A) ADDRESSEE: British American Tobacco (Investments) Limited  
 (B) STREET: Regents Park Road  
 (C) CITY: Southampton  
 (D) STATE: Hampshire  
 (E) COUNTRY: England  
 (F) POSTAL CODE: SO15 8TL

(v) **COMPUTER READABLE FORM:**

(A) MEDIUM TYPE: Diskette 3.50 inch  
 (B) COMPUTER: Viglen P5/75  
 (C) OPERATING SYSTEM: MS-DOS Windows 95  
 (D) SOFTWARE: Microsoft Word 97

(vi) **CURRENT APPLICATION DATA:**

(A) APPLICATION NUMBER: Not yet known  
 (C) CLASSIFICATION: Not yet known

(viii) **ATTORNEY/AGENT INFORMATION:**

(A) NAME: Mrs. M.R. Walford/ Mr. K.J.H. MacLean  
 (C) REFERENCE: RD-ATC-19

(ix) **TELECOMMUNICATION INFORMATION:**

(A) TELEPHONE: 01703 777155  
 (B) TELEFAX: 01703 779856

## (2) INFORMATION FOR SEQ.ID. NO:1

## (i) SEQUENCE CHARACTERISTICS

(A) LENGTH:	488 bps
(B) TYPE:	Nucleic acid
(C) STRANDEDNESS:	Double
(D) TOPOLOGY:	Linear

(ii) MOLECULE TYPE: cDNA

## (vi) ORIGINAL SOURCE:

(A) ORGANISM:	Eucalyptus grandis
---------------	--------------------

## (xi) SEQUENCE DESCRIPTION: SEQ. ID. NO:1:

ATGGGGGGGG CTTGTTGGTA TGGCAACCTG TACAGCCAAG GCTATGGCAC	50
CAACACTGCA GCTTGTGAGCA CTGCCCTGTT CAACAATGGC CTGAGCTGCG	100
GGGCATGTTA CGAGATGCGG TGCAACGACG ACCCCAGGIG GTGCCCTCCCG	150
GGGACCATCA TGGTCACGGC AACCAACTTT TGCCCTCCCA ACTTGGCCCT	200
CTCCAACGAC AATTGGGGCT GGTCGAACCC CCCCTCTCCAG CACTTCGATA	250
TGGCCGAGCC TGCTTCTTG CAGATTGCC AGTACAAAGC TGGGATTGTC	300
CAGGTTCCCT TCAGAAGGGT TCCGTGTGIG AAGAAAGGAG GGGTAAGGTT	350
CACCATCAAT GGGCACTCCT ACTTCAACTT GGTGCTGATC ACCAACGTGG	400
GAGGTGCTGG TGATGTCCAT TCCGTTCCA TCAAGGGCTC GAGGACTGGT	450
TGGCAAGCCA TGTCAAGGAA CTGGGGCAA AACTGGCA	488

(2)	<b>INFORMATION FOR SEQ. ID. NO:2</b>	
(i)	<b>SEQUENCE CHARACTERISTICS:</b>	
(A)	LENGTH:	475 base pairs
(B)	TYPE:	Nucleic acid
(C)	STRANDEDNESS:	Double
(D)	TOPOLOGY:	Linear
(ii)	<b>MOLECULE TYPE:</b>	cDNA
(vi)	<b>ORIGINAL SOURCE:</b>	
(A)	ORGANISM:	Eucalyptus grandis
(xi)	<b>SEQUENCE DESCRIPTION:</b>	SEQ. ID. NO:2:
	ATGGGGGGGG CATGCGGGTA TGGCAACCTG TACAGCCAAG GCTATGGCAC	50
	CAACACTGCA GCITTGAGCA CTGCCCTGTT CAACAATGGC CTGAGCTGCG	100
	GGCATGTAA CGAGATGCGG TGCAACGACG ACCCCCAGGTG GTGCCCTCCCG	150
	GGGACCATCA TGGTCACGGC AACCAAATTG TGCCCTCCCA ACTTGGCCCT	200
	CTCCAACGAC AATGGCGGCT GGTGCAACCC CCCTCTCCAG CACTTCGATA	250
	TGGCCGAGCC TGCCTTCTTG CAGATTGCC AGTACAAAGC TGGGATTGTC	300
	CCGGTTTCCT TCAGAAGGGT TCCGTGIGIG AAGAAAGGAG GGGTAAGGTT	350
	CACCATCAAT GGGCACTCCT ACTTCAGCTG TGGTGCTGAT CACCAACGTG	400
	GGAGGTGCTG GTGATGTCCA TTCCGTTTCC ATCAAGAGCT CGAGGACTGG	450
	TTGGCAAGCC ATGTCAAGGA ATIGA	475

(2) **INFORMATION FOR SEQ. ID. NO:3****SEQUENCE CHARACTERISTICS:**

(A) LENGTH: 494 base pairs  
 (B) TYPE: Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

(ii) **MOLECULE TYPE:** cDNA

(vi) **ORIGINAL SOURCE:**  
 (A) ORGANISM: Euclayptus grandis

(xi) **SEQUENCE DESCRIPTION:** SEQ. ID. NO:3:

ATGGGGGGGG	CATGTGGITA	CGGGGACCTT	CACAGGGCCA	CCTATGGCAA	50
GTACAGTGCC	GGCTTGAGCT	CGATGCTGTT	CAACAGAGGG	AGTACCTGCG	100
GGGCTTGCTT	CGAGCTCCGG	TGCGTCGACC	ACATTTGIG	GTGCCCTCCCT	150
GGTAGCCCGT	CGGTGATCCT	CACCGCCACC	GACTCTGCC	CTCCGAACTA	200
CGGGCTCGCG	GCAGATTACG	GCGGGTGGTG	CAACTTCCCG	CAGGAGCACT	250
TCGAGATGTC	GGAGGCGGCC	TTCGCCGAGA	TTCGGTGCG	AAGGGCTGAT	300
GTGGTGCCTA	TCCAGTACAG	GAGGGTGAAC	TGICTGAGAA	GCGGTGGTCT	350
GAGATTACA	TTCAGCGGAA	ACTCTCACTT	CTTCAGGTC	TGGTGACGA	400
ATGTAGGCCT	AGATGGGAG	GIGATTGCCA	TGAAAATGAA	GGATCGAAA	450
ACAGGGTGGA	TACCGATGGC	AAGAAACTGG	GGCAAAAAC	GGCA	494

## (2) INFORMATION FOR SEQ. ID. NO:4

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 437 base pairs  
 (B) TYPE Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Eucalyptus grandis

(xi) SEQUENCE DESCRIPTION: SEQ. ID. No. 4

ATGGGTGCC ACCGGGTCCT TGATCCTTIG ATGCCACGG AGTGCACATC	50
CCCTGCTCCG CCGACATTGG TTATGAGCAC GAGGTGAAA TAAGAATGGC	100
CGTTGACGGT GAACCGGATC CCTCCGCTTC TCCTGCACCT CACTCITCGG	150
TAGGCCACCG GGACGATCCC GGCCCTGTAC TGGCAATGT GCTGGAAGAC	200
CGGCTGGGAG AGGTCGAAAT GGAGTTGAGG AGGGTCGCAC CACCCTCCTG	250
GAGGGCAGAA GTTGGTGCC GTGACCACAA TGGCGCCCGG GAGGCACCAC	300
TGCGGGTGT TCACGCACCG GAGCTCAAAG CACGCGCCGC AGCTCAGCCC	350
ATTGTTAAC AATGCAGTGC TCAGTGCAGC TGTGTTGIG CCGTACCCCTT	400
GGCTGTATAAG ATTCCCCATAA CCACACGGCCC CCCCAT	437

## (2) INFORMATION FOR SEQ. ID. NO:5

<b>(i)</b>	<b>SEQUENCE CHARACTERISTICS:</b>				
(A)	LENGTH:	437 base pairs			
(B)	TYPE	Nucleic acid			
(C)	STRANDEDNESS:	Double			
(D)	TOPOLOGY:	Linear			
<b>(ii)</b>	<b>MOLECULE TYPE:</b>	cDNA			
<b>(vi)</b>	<b>ORIGINAL SOURCE:</b>				
(A)	ORGANISM:	Eucalyptus grandis			
<b>(xi)</b>	<b>SEQUENCE DESCRIPTION:</b>	SEQ. ID. No. 5			
ATGGGTTGCC	ACCGGGTCCT	TGATCCTTIG	ATGCCACGG	AGIGCACATC	50
CCCTGCTCCG	CCGACATTGG	TTATGAGCAC	GAGGTGAAA	TAAGAATGGC	100
CGTTGACGGT	GAACCGGATC	CCTCGCTTC	TCCTGCACCT	CACTCTCGG	150
TAGGCCACAG	GGACCGATCCC	GGCCCTGTAC	TGCGCAAATGT	GCTGGAAGAC	200
AGGCTGGGAG	AGGTGAAAT	GGAGTTGAGG	AGGGTCGGAC	CACCCCTCCTG	250
GAGGGCAGAA	GTGGTGGCC	GTGACAACAA	TGGCGCCCGG	GAGGCACCAC	300
TGCGGGTCTG	TCACGCACCG	GAGCTCAAAG	CACGCGCCCG	AGCTCAGCCC	350
ATTGTGAAC	AATGCAGTGC	TCAGTGCAGC	TGTGTTGIG	CCGTACCCCTT	400
GGCTGTATAG	ATTCCCATAA	CCACACGCC	CCCCCAT		437

## (2) INFORMATION FOR SEQ. ID. NO:6

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 488 base pairs  
 (B) TYPE Nucleic acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Eucalyptus grandis

(xi) SEQUENCE DESCRIPTION: SEQ. ID. No. 6

CCTTGACATG GTCGCCACC TTGTCGGCGA ACCCTTCACG GCGACCGAGT	50
TGACGTTGCC TGGCCCGCCG ACGTTTGTGA CGAGGGACGAG CTTGAAGTAT	100
GAGTTGCCGT TGATGGGAA CCGGATGCCT CCTCTCCTCC TGCACGTAC	150
CCTCCGTGAC GCAACGTGGA CGATGCCGGC TCGGTACTTG GCAATGTGCT	200
GGAAGACGGG CTGGGAGATG TCGAAGTGGT GTTGGGGCGG GTTGCACCAT	250
CCGCCGGCGT TGTTGGGAG GGC GTTGT TT GGCGGGCAGA AGITIGTG	300
GGTGACGACG ATGGAGCCGC CCAGGCACCA CTTCGGTCG TTCA CGCACC	350
GGATCTCGAA GCACGACCCC CAGCTCAGCC CGTTTTTAA CAGCGCCGTG	400
CTCAGCGCCG CGTGTTCGT ACCGTAGCCC TGGCIGTACA GGTGCG	448

## (2) INFORMATION FOR SEQ. ID. NO:7

- (i) SEQUENCE CHARACTERISTICS:
- |                   |                |
|-------------------|----------------|
| (A) LENGTH:       | 19 nucleotides |
| (B) TYPE          | Nucleic acid   |
| (C) STRANDEDNESS: | Single         |
| (D) TOPOLOGY:     | Linear         |
- (ii) MOLECULE TYPE: Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ. ID. No. 7

ATGGIGGIGC NTGTGGNTA

19

Key I = Inosine

N = A, G, T, or C

## (2) INFORMATION FOR SEQ. ID. NO:8

- (i) SEQUENCE CHARACTERISTICS:
- |                   |                |
|-------------------|----------------|
| (A) LENGTH:       | 20 nucleotides |
| (B) TYPE          | Nucleic acid   |
| (C) STRANDEDNESS: | Single         |
| (D) TOPOLOGY:     | Linear         |
- (ii) MOLECULE TYPE: Synthetic DNA
- (xi) SEQUENCE DESCRIPTION: SEQ. ID. No. 8

TGCCARTTYT GNCCCCARTT

20

Key R = A or G

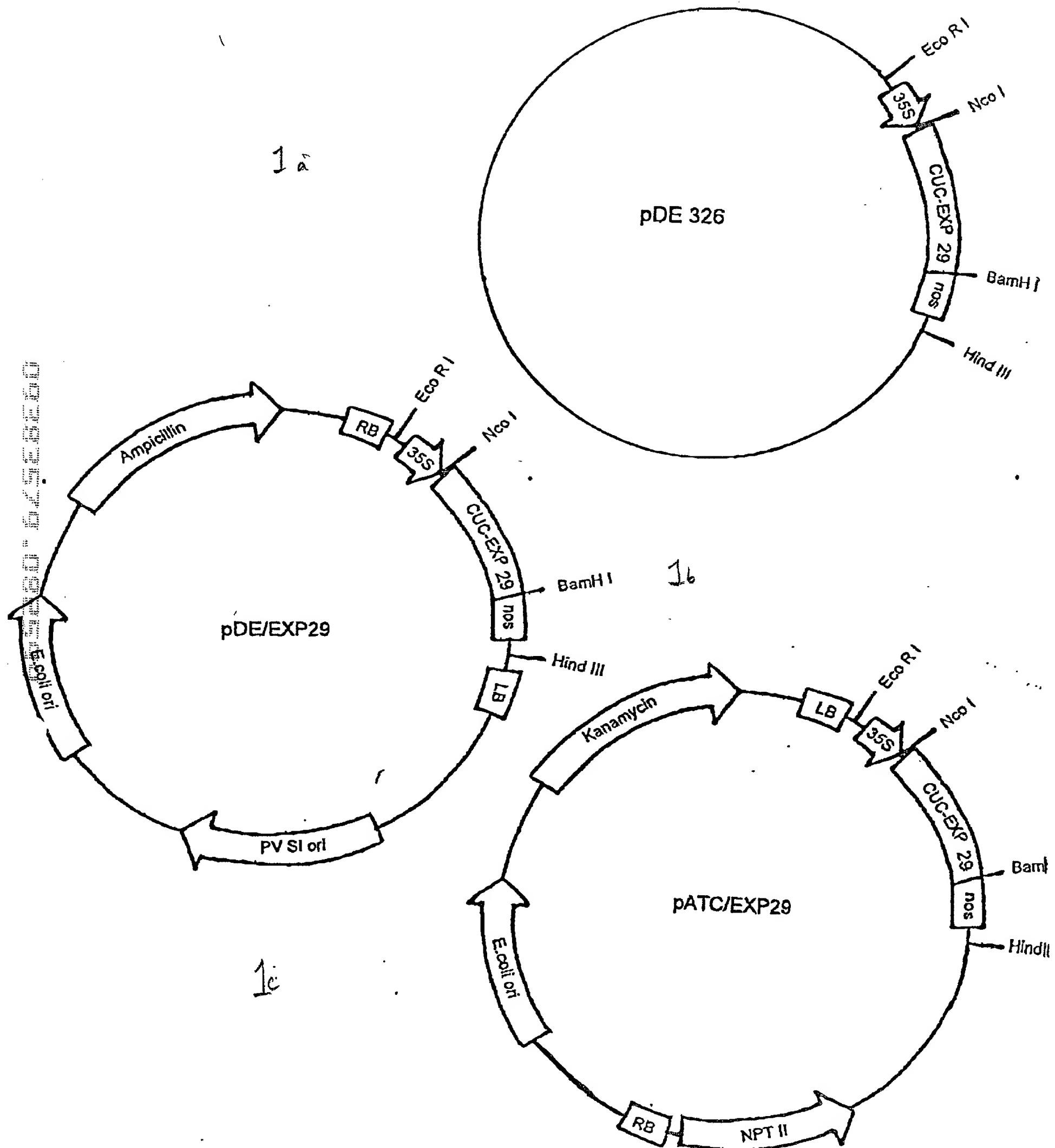
Y = T or C

N = A, G, T or C

9341 - 018

1 of 2

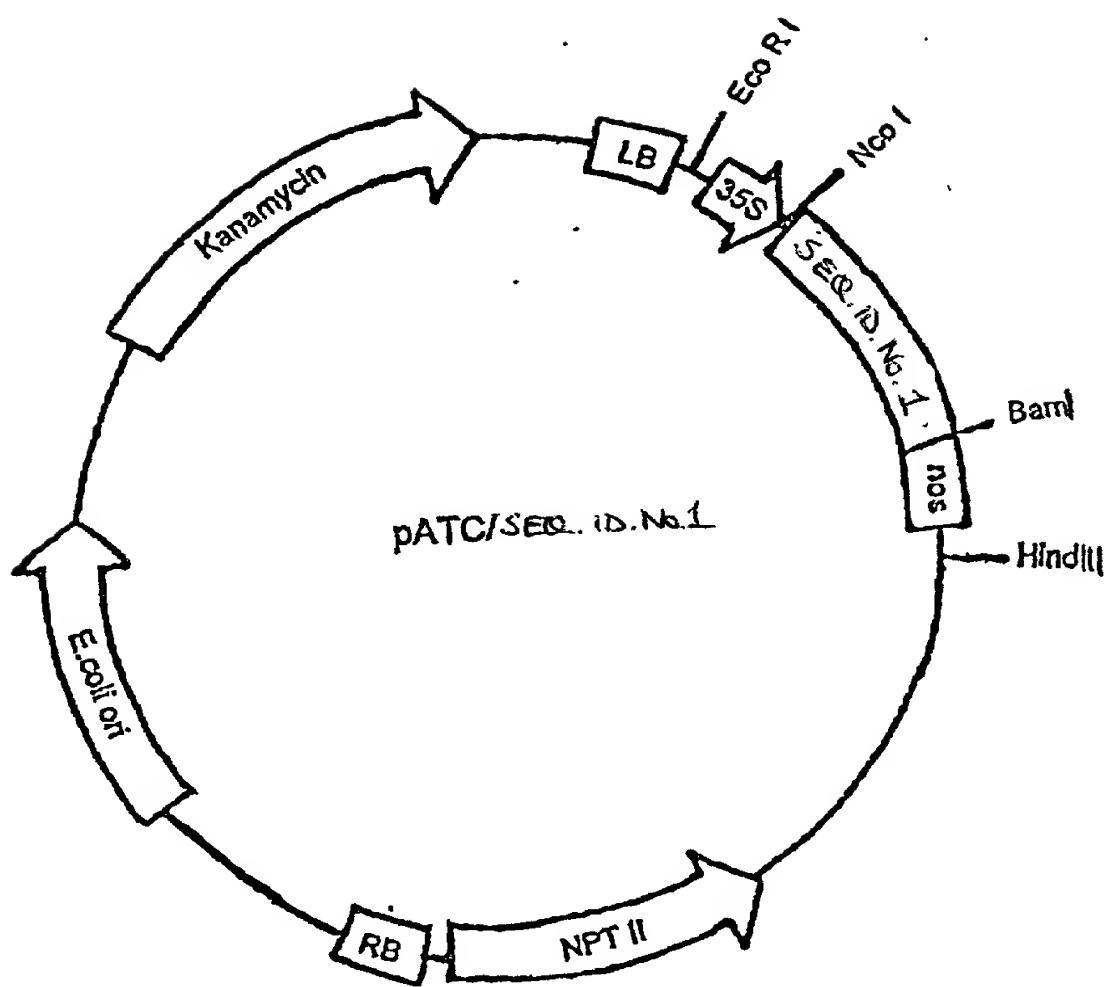
Figure 1



9341 - 018

2 ♂ 2

Figure 2



**DECLARATION  
AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

**Modification of Plant Fibres**

and for which a patent application:

- is attached hereto and includes amendment(s) filed on *(if applicable)*
- was filed in the United States on as Application No. *(for declaration not accompanying application)* with amendment(s) filed on *(if applicable)*
- was filed as PCT international Application No. on and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
9818808.9	United Kingdom	29 August 1998	YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

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